EXHIBIT "B"

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Use of RT-PCR and DNA Microarrays to Characterize RNA Recovered by Non-Invasive Tape Harvesting of Normal and Inflamed Skin

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We describe a non-invasive approach for recovering RNA from the surface of skin via a simple tape stripping procedure that permits a direct quantitative and qualitative assessment of pathologic and physiologic biomarkers. Using semi-quantitative RT-PCR we show that tape-harvested RNA is comparable in quality and utility to RNA recovered by biopsy. It is likely that tape-harvested RNA is derived from epidermal cells residing close to the surface and includes adnexal structures and present data showing that tape and biopsy likely recover different cell populations. We report the successful amplification of tape-harvested RNA for hybridization to DNA microarrays. These experiments showed no significant gene expression level differences between replicate sites on a subject and minimal differences between a male and female subject. We also compared the array generated RNA profiles between normal and 24 h 1% SLS-occluded skin and observed that SLS treatment resulted in statistically significant changes in the expression levels of more than 1,700 genes. These data establish the utility of tape harvesting as a non-invasive method for capturing RNA from human skin and support the hypothesis that tape harvesting is an efficient method for sampling the epidermis and identifying select differentially regulated epidermal biomarkers.

Key words: tape strip/dermatis/inflammation/DNA microarray/quantitive RT-PCR/epidermis/expression profile
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Contact dermatitis, a common skin reaction, involves several signaling pathways. Irritant contact dermatitis (ICD) predominantly involves keratinocyte activation (Freedberg *et al*, 2001), whereas Langerhans' cell presentation of antigen to T cells in draining lymph nodes and recognition of the offending allergen in skin by memory T cells control the initiation and expression of allergic contact dermatitis (ACD; Feghali and Wright, 1997) Clinically, both contact dermatitides are characterized by pruritus, erythema, and edema. This commonality of the clinical signs and symptoms makes distinguishing between ICD and ACD difficult at the clinical level, particularly when symptoms are subtle. By contrast, at the molecular level, ICD and ACD are believed to be characterized by unique mRNA patterns, although the published literature is conflicting (Hoefakker *et al*, 1995; Filer *et al*, 1999; Morhenn *et al*, 1999; Ryan and Gerberick, 1999; Ulfgren *et al*, 2000; Cumberbatch *et al*, 2002). Documentation of simple and complex mRNA profiles is possible using RT-PCR and DNA microarray technologies. Using the technique of tape stripping, RNA can be harvested from both normal and inflamed skin (Morhenn *et al*, 1999) and by combining tape stripping and RNA profiling, it may be possible to non-invasively establish a diagnosis of ICD or ACD.

In this study, we expand upon the work of Morhenn *et al* (1999) who used adhesive tape as a substitute for punch biopsy to non-invasively sample the epidermis. Morhenn *et al* demonstrated that when a skin site was tape stripped 20 or more times, it was possible to recover sufficient RNA from skin cells adherent to the tapes to detect and semi-quantify specific mRNAs using the ribonuclease protection assay. We demonstrate in this work that sufficient RNA can be recovered using sequential application of as few as four small tapes.

In order to document the use of tape harvesting as an accurate and reliable sampling method we performed a clinical trial in which occlusive patches containing either 1% SLS (irritant) or water (vehicle control) were applied to the mid-back of 10 subjects for 24 h. The sites were then clinically assessed and, along with normal control skin, surface cells were harvested with four applications of individual tapes and by shave biopsy. RNA was extracted from the tapes and biopsies and assayed semi-quantitatively for IL-1β, IL-8, GAPDH, and β-actin mRNA using fluorescent, quantitative RT-PCR. The results showed consistent increases in IL-1β and IL-8 mRNA in inflamed skin relative to untreated skin. We further report the successful use of tape-harvested RNA to profile normal

Abbreviation: PPDE, posterior probability of differential expression; SLS, sodium lauryl sulfate

and experimentally inflamed skin using DNA microarrays. This profile of SLS-irritated skin is the first step in the definition of RNA profiles designed to differentiate irritant from allergic skin reactions.

Results

Total RNA yields RNA was recovered from 27 of 30 skin sites using four tapes as described in Materials and Methods. The amount of total RNA recovered was variable from site to site and subject to subject (data not shown). The average mass of RNA recovered from normal skin sites was 0.92 ng (± 0.35) with a range of 0 (two samples)–3.2 ng. The average mass of RNA recovered from water-occluded skin was 0.69 ng (± 0.27) with a range of 0 (one sample)–2.7 ng. SLS inflamed skin produced the greatest average yield of RNA with an average of 185 ng (± 76) and a range of 0.067–747 ng.

Relative levels of housekeeping genes in tape-strip samples and biopsies We have chosen as markers of the inflammatory process IL-1β and IL-8 mRNAs. We have accounted for differential recovery of total RNA mass in a sample by normalizing these mRNAs to an internal control, the β-actin transcript. We then calibrated this normalized RNA ratio to the analogous ratio in a control sample (normal skin) using the comparative or $\Delta\Delta C_t$ method (described in Materials and Methods). In this study, IL-1β and IL-8 mRNA are predicted to increase relative to β-actin in response to SLS treatment, whereas the level of housekeeping mRNAs, such as β-actin and GAPDH are assumed to remain constant. We have tested the assumption that housekeeping mRNAs such as GAPDH and β-actin are unchanging among different samples by measuring the relative ratio of these two mRNAs.

If the relative ratio of two housekeeping genes is unchanged in different samples, the relative ratio of GAPDH/β-actin mRNA between two samples should be equal to 1. The data in Table I reveal the fold-change of the GAPDH/β-actin mRNA ratio in SLS- and water-treated samples relative to normal skin and also water-treated skin. The tape sample data show that the average fold-change in SLS samples was 0.55, whereas the average fold-change in water-treated samples was 1.14. Biopsy samples showed similar and minor changes with SLS-treated samples having an average 0.39 fold-change, whereas water-treated samples have an average 0.53 fold-change. Individual subject data are shown in Online Table S1. Although there are some examples of statistically significant changes relative to the normal skin, these changes are not sufficient to explain the fold-changes in IL-1β and IL-8, which are reported in Table I. Thus, it is likely that our housekeeping genes do change relative levels in response to SLS and water treatment, but the magnitude of those changes is minor. Similar observations and conclusions have been reported by Paludan and Thestrup-Pedersen (1992) and Grangsjo *et al* (1996).

IL-1β/β-actin mRNA ratios in SLS-irritated and control skin Data in Table I reveal the average fold-change of the IL-1β/β-actin mRNA ratio in water-occluded and SLS-occluded skin relative to normal skin, in tape- and biopsy-harvested RNA samples. Individual subject data can be found in Online Table S2. Table I shows that on average the IL-1β/β-actin mRNA ratio in biopsy samples of SLS-treated skin was elevated at least 11-fold (>95% confidence interval) compared with normal skin. Furthermore, the IL-1β/actin ratio was 4.42-fold elevated in SLS- relative to water-treated samples. In six of 10 subjects water occlusion produced significant (>95% confidence) increases in the IL-1β/actin ratio but this increase was typically 2–4-fold and was always smaller than the respective effect of SLS occlusion (Online Table S3). Thus SLS-occlusion produced the most consistent elevation of the IL-1β/actin mRNA ratio but water-occlusion did effect a similar albeit smaller response.

Table I. Summary of fold-change in GAPDH, IL-1β, and IL-8 mRNA relative to β-actin mRNA in SLS-treated and water-treated skin calibrated to control skin

		Average fold-change by sampling method and treatment[a]			
		Tape		Biopsy	
mRNA	Calibrator[b]	Water	SLS	Water	SLS
GAPDH	Normal	1.14 (0.6–2.14)	0.55 (0.31–0.99)	0.53 (0.43–0.66)	0.39 (0.29–0.53)
	Water	1	0.48 (0.36–0.65)	1	0.74 (0.55–1)
IL-1β	Normal	ND	>2.66	>2.49	>11
	Water	1	>1.5	1	4.42 (1.42–13.76)
IL-8	Normal	1.94 (0.63–5.98)	10.32 (3.89–27.34)	>1.54	>52.45
	Water	1	5.31 (2–14.1)	1	34.06 (6.37–181.87)

[a]Average fold-change in up to 10 subjects (n = 8 for normal skin tape samples, n = 9 for water-treated skin tape samples and n = 10 for all others). The average fold-change is calculated from the subject-average ΔC_t values given in Online Tables S3, S4, S7. Individual fold-change values are reported in Online Tables S1, S2, S5. Values preceded by > are lower limit estimates necessitated by the fact that the applicable mRNA was not detectable in the control (calibration) samples. The >95% confidence interval for fold-change is given in parenthesis.
[b]SLS and water samples are calibrated to normal skin samples; additionally, SLS samples are calibrated to the water sample. Calibration is described in Materials and Methods.

Data in Table I also reveal the fold-change of IL-1β/β-actin mRNA in tape-harvested samples of water-occluded and SLS-occluded skin relative to water-occluded and normal skin. Individual subject data are shown in Online Tables S2 and S3. In most subjects, IL-1β was undetectable in normal and water-treated, tape-harvested skin samples. The average fold-change of the IL-1β/actin ratio was estimated to be at least 2.66-fold in SLS-treated samples. Although the average fold-change for eight subjects was modest, five of these subjects showed significant (>95% confidence) IL-1β/actin ratio increases that were in qualitative agreement with the biopsy data (Online Table S3). Analysis of the remaining five samples was indeterminate because IL-1β mRNA was not detected in either the water or normal skin sample.

IL-8/β-actin mRNA ratios in SLS-irritated and control skin Table I reveals the fold-change of the IL-8/β-actin mRNA ratio in water-occluded and SLS-occluded skin relative to normal skin. The data demonstrate that the IL-8/actin ratio was on average 52-fold elevated in SLS-treated skin samples relative to normal skin and 34-fold elevated relative to water-treated skin. Biopsy samples from nine of 10 untreated skin sites had undetectable IL-8 mRNA levels and the sole normal skin biopsy sample with detectable IL-8 mRNA was close to the level of detection (Online Tables S4 and S5). Thus IL-8 mRNA was generally not detectable in a biopsy of normal skin.

Table I reveals that the IL-8/actin mRNA ratio was on average 10-fold increased in tape-harvested samples from SLS-occluded sites (>95% confidence interval) and 1.94-fold increased in water-treated samples (not significantly different than normal skin). We conclude that the tape data are in good qualitative agreement with the biopsy data, with a majority of inflamed sites revealing increases in IL-8 mRNA.

During our analysis of IL-8 mRNA in biopsy and tape samples, we observed that although the fold-change was in qualitative agreement between the two methods, the primary ΔC_t data were strikingly different (Online Table S4). As described in Materials and Methods, ΔC_t is a measure of the ratio of two mRNAs in a sample. Online Table S4 shows that the $\Delta C_{t,IL\text{-}8}$ for tape samples is very different than that of biopsy samples—for both SLS- and water-treated skin sites—and this difference is highly significant (both p-values <0.005). The most striking example of this is revealed by a comparison of the average $\Delta C_{t,IL\text{-}8}$ in tape-harvested samples compared with biopsy-harvested samples of water-occluded sites. For tape-harvested samples the average $(\Delta C_{t,IL\text{-}8})_{water}$ is 1.54, whereas for biopsy samples it is 9.22 (Online Table S4). The data suggest that in the cell population harvested by tape, IL-8 mRNA is much more abundant relative to β-actin mRNA than in biopsy samples. The average relative abundance can be estimated as equal to $2^{-(1.54-9.22)}$ or 205. In this calculation, the biopsy sample acts as the "calibrator", thus the tape samples have, on average, a 205-fold greater IL-8/actin mRNA ratio than biopsies. A similar calculation can be performed with the SLS sample data (Online Table S4), which also reveals a highly significant difference between tape and biopsy $(\Delta C_{t,IL\text{-}8})_{SLS}$ values ($p<0.005$). We conclude from this data that tape harvesting recovers a distinctly different cell population than does biopsy.

DNA microarray analysis of RNA extracted from normal skin using tape The success in the above analysis of tape-harvested RNA from different skin sites suggested that this RNA might be amenable to amplification and hybridization to DNA microarrays. In order to assess the reproducibility and consistency of tape-harvested RNA samples for gene expression profiling experiments, we collected three samples from the upper back of each of two healthy individuals, one male (sample C1, C2, and C3) and one female (sample A5, A6, and A9). Approximately 1 ng of total RNA was isolated and the mRNA was amplified and biotin labeled using a MessageAmp aRNA kit (Ambion Inc., Austin, Texas) as described in Materials and Methods. The resulting biotin-labeled aRNA from each sample was used for hybridization to an Affymetrix HG-U133A GeneChip.

The results in Table II show the differences observed when a matrix of pairwise gene expression comparisons between two GeneChips was performed using Affymetrix Microarray Suite software. These data show an average of only 12% variance among gene measurements, regardless of whether data from different sites on the same individual or sites from different individuals are compared. Furthermore, comparing the data in quadrant three of Table II (A *versus* C) to the data in quadrants one (A *versus* A) and four (C *versus* C) shows that about 15% of this variance is due to either gender difference (A *versus* C) or inter-subject variation (A *versus* A or C *versus* C). Thus, amazingly little variance is contributed by samples obtained from different sites or from different individuals.

To compare these data in a more quantitative manner, the three Affymetrix GeneChips each hybridized with targets from RNA samples obtained from individual A were compared with three GeneChips hybridized with targets from the three RNA samples obtained from individual C. These data were analyzed with a regularized *t* test (Long *et al*, 2001) implemented in the Cyber-T statistical program. This three-by-three comparison revealed 21,790 probe sets

Table II. Percentage of the measurement of gene expression reported as unchanged by the Affymetrix MAS 5.0 software for each of all possible pairwise comparisons among GeneChips (A5, A6, A9, C1, C2, and C3) hybridized with aRNA obtained from three different locations on the upper back of two subjects (A and C)

	GeneChip/subject ID (%)					
	A5	A6	A9	C1	C2	C3
A5	100					
A6	88.90	100				
A9	90.80	86.10	100			
C1	89.80	88.20	87.40	100		
C2	86.00	85.90	83.10	89.60	100	
C3	88.00	88.00	87.30	88.90	83.70	100

that exhibited gene expression levels above background for all three sites from each subject. Of these genes 1117 (5%) were differentially expressed with p-value less than 0.0035, which based on the global false positive and negative levels of this dataset corresponds to a PPDE value of 0.95. Thus, 56 of the 1117 differentially expressed genes that exceed this p-value threshold are expected to be false positives. The source of these inter-subject gene expression differences remains to be determined; however, at least one of these differences is gender based. For example, the gene with the smallest p-value and the highest PPDE value is the Y-linked ribosomal protein S4 (PRS4Y). It is likely that differences that are not gender based are a reflection of normal variation of gene expression between individuals. These data are available at www.igb.uci.edu.

DNA microarray analysis of normal versus water-occluded and SLS-occluded skin In a separate experiment, a total of nine RNA samples ranging from 1 to 10 ng were isolated by tape harvesting from three untreated, three water-occluded, and three SLS-occluded sites of each of three individuals. mRNA from each of the nine samples was amplified, biotin labeled and used for hybridization to each of nine Affymetrix HG-U133A GeneChips as shown in Fig 1.

Untreated versus SLS-treated samples A comparison of gene expression levels between three untreated (A1, B1, C1) samples and three SLS-treated (A2, B2, C2) samples revealed 21,031 genes that exhibited expression levels above background for all samples. To assess the confidence in global changes in gene expression, methods implemented in Cyber-T were used to determine the posterior PPDE of each gene based on experiment-wide global false positive and negative gene measurement levels as described by Hung *et al* (2002) and Baldi and Hatfield (2002). When untreated *versus* SLS-occluded data are compared, the p-values for the differentially expressed genes are low and cluster toward 0. This is consistent with highly statistically significant differences among measurement levels of some genes. In fact 1771 genes are differentially expressed with a threshold of $p = 0.003$, which corresponds to an experiment-wide probability for differential expression (PPDE value) equal to or greater than 0.99. These data are available at www.igb.uci.edu.

SLS- versus water-treated samples A comparison of gene expression levels between three SLS-treated (A2, B2, C2) samples and three water-treated (A3, B3, C3) samples revealed 21,307 genes that exhibited expression levels above background for all samples. Based on a threshold of $p = 0.003$, 1364 genes are differentially expressed with a PPDE value of 0.99. Of these, 1063 genes are also differentially expressed with a p-value of 0.003 and a PPDE value of 0.99 when SLS and untreated samples are compared. These data are available at www.igb.uci.edu.

Untreated versus water-treated samples A comparison of gene expression levels between three untreated (A1, B1, C1) samples and three water-treated (A3, B3, C3) samples revealed 21,164 genes that exhibited expression levels above background for all samples. This comparison revealed no statistically significant differential expression. Nevertheless, based on our review of the genes assigned the lowest p-values, many of which are associated with inflammation, we believe that the water treatment does lead to some changes in gene expression compared with untreated control skin. These data are available at www.igb.uci.edu.

For purposes of discussion, only the 100 genes differentially expressed with p-values less than 1.4×10^{-10} and PPDE values greater than 0.99 are discussed here, in Fig 2 and are fully described in Online Table S6. An examination of these top 100 genes most significantly altered when the SLS-treated skin samples were compared with untreated skin samples revealed that, as expected, most of these genes carry out functions related to tissue inflammation and injury (Fig 2; Online Table S6). These differentially expressed genes are proteinases, protease inhibitors, cytokines, chemokines, complement components, HLA factors, or receptors involved in immune regulation. These associations with inflammation and injury responses for many of these mostly upregulated genes are documented in the literature (Online Table S6). These results demonstrate that the tape-stripping method described here harvests RNA suitable for complete gene expression profiles of the skin that accurately reflect its pathological state.

Discussion

Recent advances in molecular medicine have made the possibility of molecular diagnosis a reality (Altman, 2001; Bertucci *et al*, 2001; Gallegue and Casellas, 2002; Lacroix *et al*, 2002; Whipple and Kuo, 2002; Satagopan and

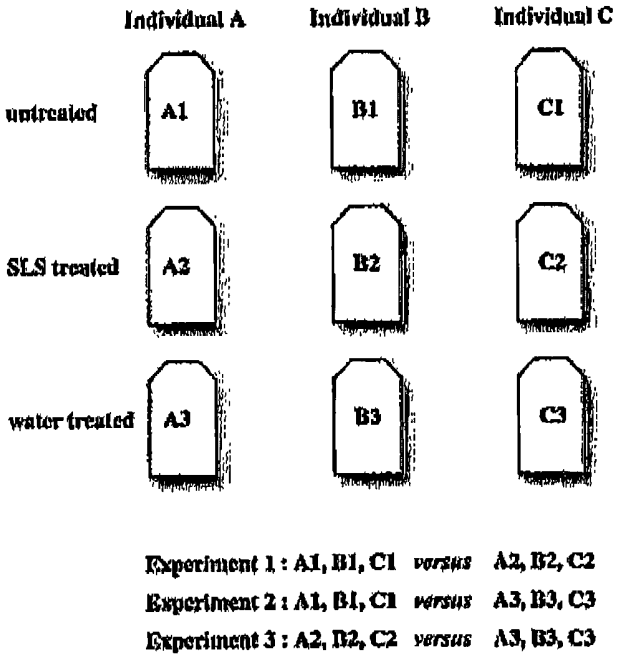

Figure 1
Experimental design of SLS irritation protocol. Details are described in the text.

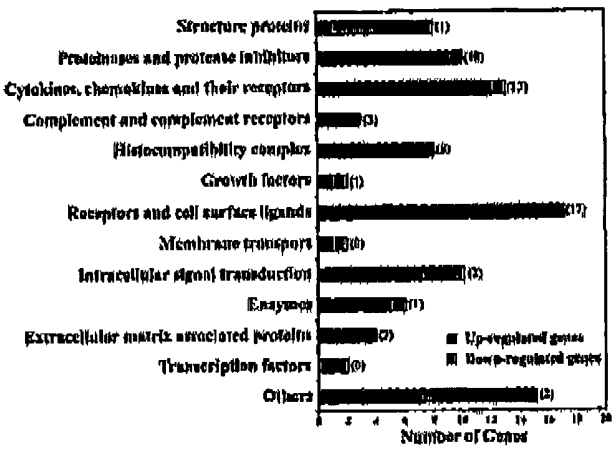

Figure 2
Functional grouping of the top 100 differentially expressed genes between untreated and SLS-treated skin with p-values less than 1.4×10^{-19} and PPDE values greater than 0.99. Genes that are upregulated in SLS-treated conditions are represented with dark gray color and genes that are downregulated in SLS-treated conditions are represented by light gray. The number in parentheses is the number of genes in each functional group that has been previously documented to be related to the inflammatory response.

Panageas, 2003). Through the use of microarrays and RNA profiling it is becoming increasingly clear that simple and complex cell populations can be monitored or "profiled" with the intent of understanding the physiologic state of those cells or tissues. This information is expected to lead to more accurate and possibly predictive diagnoses. We have shown here that the use of four small tape strips is an effective and basically non-invasive approach to capturing messenger RNA from the surface of skin and that this technique permits a direct quantitative and qualitative assessment of pathologic and physiologic biomarkers as a function of normal physiology.

We have assayed semi-quantitatively the levels of IL-1β and IL-8 mRNA relative to β-actin in normal, water and SLS-occluded skin sites and shown that RNA from tape and biopsy samples produce qualitatively similar results. In order to account for the possibility that changes in β-actin mRNA were responsible for observed changes in the interleukin/β-actin mRNA ratios (Suzuki *et al*, 2000; Bustin, 2002; Tricarico *et al*, 2002) we quantified the levels of two housekeeping genes relative to each other. The resulting data (Table I) showed that although the GAPDH/β-actin mRNA ratio is different in differently treated skin samples, the magnitude of this difference is not capable of explaining the observed changes in IL-1β and IL-8 mRNA levels induced by SLS treatment. This fact is most clearly demonstrated by tape-harvest and biopsy data in which IL-1β and IL-8 mRNA are virtually undetectable in control samples but easily detected in SLS-treated samples, an observation that cannot be explained by minor changes in β-actin mRNA levels. In addition, IL-1β and IL-8 mRNAs and proteins have been well characterized in inflammation and are known to become elevated in response to SLS and other treatments (Paludan and Thestrup-Pedersen, 1992; Grangsjo *et al*, 1996; Corsini and Galli, 1998; Tomic-Canic *et al*, 1998; Freedberg *et al*, 2001; Perkins *et al*, 2001; Cumberbatch *et al*, 2002; Coquette *et al*, 2003). Thus our data, from both tape and biopsy, are consistent with published observations.

Biopsy and tape harvesting are not equivalent sampling methods, and therefore, should not be expected to yield identical results. Tape harvest is restricted to the skin surface, and therefore, may preferentially recover vellus hair follicles and cells lining sebaceous, eccrine and sweat ducts as well as corneocytes (not predicted to contain RNA). Our method of using a single application of four individual tapes does not result in glistening of normal skin and thus does not bare the viable epidermis. In contrast, a shave biopsy is expected to include not only cells of the epidermis (primarily keratinocytes, melanocytes, and immune cells) but fibroblasts from the upper dermis. The potential enrichment of surface epidermis and adnexal structures conveyed by our circular tape compared with a shave biopsy can be appreciated by considering that the surface area of a tape is 284 mm^2, whereas the surface area of a 2 × 2 mm shave biopsy is 4 mm^2.

The hypothesis that tape and biopsy recover different cell populations is supported by the $\Delta C_{t,IL\text{-}8}$ data previously discussed and presented in Online Table S4. Similarly, supportive data are presented in Online Table S7 for $\Delta C_{t,GAPDH}$ values. Thus we propose that tape-harvested cells represent an enrichment of a subpopulation of cells found in a shave biopsy. This data imply that some differentially expressed biomarkers may be best detected in tape rather than biopsy-harvested epidermal samples.

Identification of biomarkers diagnostic of clinical irritation has been a long sought goal (Muller-Decker *et al*, 1994; Boelsma *et al*, 1998; Muller-Decker *et al*, 1998; van Ruissen *et al*, 1998; Komine *et al*, 2001; Perkins *et al*, 2001; Boxman *et al*, 2002; Perkins *et al*, 2002; Coquette *et al*, 2003). We have used changes in IL-1β and IL-8 mRNA as indicators of irritation and shown that most but not all irritated sites display increased levels of these normalized mRNA markers (Table I and Online Tables S2 and S5). Our data also show that tape-harvested and biopsy-recovered RNA are qualitatively equal in their ability to reveal an irritant skin reaction. With respect to biopsy samples, it is evident that neither marker is 100% efficient at diagnosing irritation, a result observed for every biomarker proposed to be diagnostic of erythema and inflammation (Grangsjo *et al*, 1996; Muller-Decker *et al*, 1998; Chung *et al*, 2001; Perkins *et al*, 2001; Boxman *et al*, 2002). The current limitation of the tape-harvest assay is the inefficiency in detecting certain markers in samples with limiting amounts of RNA, a subject discussed below. But in comparison with the Sebutape assay (immunoassay of IL-8 protein; Perkins *et al*, 2001) for irritation, which has a sensitivity (Hoffrage *et al*, 2000) of approximately 30%, mRNA biomarkers seem to possess superior potential. Our observation that water occlusion produced increases of our biomarker ratios in some subjects has been reported by others (Grangsjo *et al*, 1996; Howie *et al*, 1996; Perkins *et al*, 2001).

It is reasonable to assume from the data presented here and in the literature that singular or limited multiplex assays will not be sufficiently predictive or diagnostic of irritation. Since our results show that the tape-stripping method

harvests RNA suitable for DNA microarray experiments, and that these gene expression profiles reflect the pathological state of human skin, however, it should be possible to identify a subset of genes whose differential expression patterns can be correlated with different pathological states with a high degree of statistical accuracy. The fact that 1700 differentially expressed genes have been identified with high statistical confidence sets the stage for the creation of small custom DNA arrays designed to identify patterns of gene expression diagnostic of irritant skin reactions, possibly diagnostic of different irritants and predictive of irritant or toxic reactions. The next step along this path is to identify the analogous set of genes expressed during an allergic skin response, identify genes unique to the irritant or allergic response and combine them into one DNA array, which could be used to determine if a mild reaction to a substance is irritant or allergic in nature. Such an array could also be used to test a variety of irritants and allergens for unique profiles.

Analysis of the top 100 genes differentially expressed in our SLS-treated samples shows that well over half of these genes have been implicated in injury and inflammation (Fig 2; Online Table S6), with most of these genes being upregulated. The DNA array data also confirm our RT-PCR data with IL-1β being found in the top 100 most significantly differentially expressed (p-value less than 3.56×10^{-11}, probability of differential expression (PPDE) value >0.99) genes. Although the IL-8 gene was not among the top 100 most significantly differentially expressed genes, it was ranked number 395 in the normal *versus* SLS-treated comparisons (p-value $= 6 \times 10^{-8}$, PPDE $= 0.99$) and ranked number 183 (p-value $= 5 \times 10^{-8}$, PPDE $= 0.99$) in the SLS-treated *versus* water-treated comparisons. The expression level of IL-1β was elevated 11-fold and IL-8 was elevated 8-fold in the SLS-treated sample. Interestingly, many of the downregulated genes are hair keratins and keratin associated proteins selectively expressed in the hair during the anagen phase of the hair cycle. Either, the occlusive SLS treatment removes hair prior to the tape stripping or the treatment blocks anagen in the hair follicles.

We have shown that RNA can be non-invasively and productively recovered from the surface of the skin using four small tape strips. The number of tape strips can be reduced to two in conditions where the surface of the skin has been disrupted, such as SLS occlusion for 24 h (Nicholas Benson, personal communication). Furthermore, the limitation of capturing small amounts of RNA from some skin sites can be effectively overcome by obtaining replicate control samples (data not shown) and by the appropriate choice of mRNA biomarker (discussed below). We have also presented data that the $\Delta C_{t,IL\text{-}8}$ value, which is normally used to calculate a $\Delta\Delta C_t$ value (calibrated fold-change), has revealed that tape and biopsy recover different cell populations.

In this study, the quantity of RNA recovered from different individuals and skin sites was variable, with significantly more RNA being recovered from SLS-treated sites than normal skin sites. The large amount of RNA recovered from the SLS-irritated sites is consistent with the known effects of SLS, which effects invasion of inflammatory cells and creates a weakened barrier facilitating the removal of the inflamed epidermis. We have since shown that recovery of RNA from normal skin is also a function of anatomical site and similar sites vary between individuals with respect to RNA yield (data not shown).

Although the variability of total RNA recovered does not affect the results of relative gene quantitation, the recovery of very small amounts of RNA did affect our ability to fully analyze some samples. In this respect, the choice of biomarkers may be as important as the amount of RNA recovered from a site. For instance, Online Table S7 shows that most tape-harvested samples could be assayed for β-actin and GAPDH mRNAs and thus calibrated GAPDH/actin ratios could be calculated (Online Table S1). Online Tables S2 and S5 reveal, however, that some of these same samples do not have calibrated IL-1β/actin or IL-8/actin mRNA ratios, with the IL-1β assay being the most affected. The reason for this difference between biomarker assays lies in the relative abundance of the specific mRNA. Because GAPDH mRNA is approximately equal in abundance to β-actin mRNA, all samples with detectable actin mRNA were successfully assayed for GAPDH. Likewise, we were highly successful at calibrating IL-8/actin mRNA ratios in water and SLS treated tape-harvested skin samples because IL-8 message is relatively abundant in these samples. Thus the biomarker mRNA that is the most abundant relative to the normalizing mRNA will make the most efficient use of RNA mass. Therefore, candidate biomarker mRNAs should be chosen for best sensitivity, positive predictive value and high relative abundance when RT-PCR is to be used for detection and tape harvesting is to be the sampling method.

We have chosen to demonstrate the utility of tape-harvested RNA for semi-quantitative RT-PCR and microarray applications for several reasons. Both methods have particular advantages and are appropriate in different circumstances. The use of microarrays is an invaluable tool for the discovery of diagnostic and prognostic biomarker candidates and may be essential for subcategorizing disease states, which may demand simultaneous assay of hundreds of biomarkers. The use of microarrays, however, is expensive and technically laborious. Quantitative RT-PCR is less expensive and less technically demanding and is appropriate for studies where a limited number of known markers are being studied.

In summary, our data show that the tape-stripping method non-invasively collects skin samples from normal and inflamed skin that are suitable for RNA isolation and gene expression profiling experiments. In the future, this method can be used to profile expression of a large number of genes in different skin conditions with the goal of designing custom arrays that allow molecular diagnoses of skin disorders.

Materials and Methods

Clinical protocols The study protocols were reviewed and approved by an independent IRB (BioMed IRB, San Diego, California), all subjects signed informed consent and protocols adhered to the Declaration of Helsinki Guidelines. Ten healthy women, ages 21–55 were enrolled in the study. Regions of unblemished, normal appearing skin on the mid-back were chosen for the application of two occlusive patches in the form of bandages approximately 4 cm × 6.5 cm. The bandages were

made using a clear, non-porous plastic hypoallergenic adhesive tape. In the center of this tape was a Webril (non-woven cotton) patch measuring approximately 2 cm × 4.5 cm. One Webril patch contained 0.6 mL of 1% aqueous sodium lauryl sulfate and the other contained 0.6 mL of sterile water as the vehicle control. Patches were arranged such that the SLS patch was superior and directly adjacent to the water patch; the area of normal control skin was inferior to and adjacent to the water patch. At 24 h post application, the SLS and water patches were removed and the skin allowed to air dry for 15 min before scoring. The sites were scored by a trained technician using the scale provided below. Patched sites were large enough that two areas could be tape harvested without overlap and room left for a shave biopsy (~ 2 × 2 mm). Skin sites were tape stripped with four tapes each; shave biopsies were taken under local anesthetic (lidocaine HCl 1% and epinephrine 1:100,000; Abbott Laboratories, Abbott Port, Illinois). An area of normal skin was similarly tape harvested and shave biopsied. Tapes were stored in individual eppendorf tubes at −80 until extraction; biopsy samples were placed in buffer RLT and stored at −80 until extraction. Skin responses to each patch application were examined and graded under light supplied by a 100-W incandescent blue bulb. The following grading scale was used: 0, no visible reaction; 1, slight, pink, patchy erythema; 2, mild confluent, pink erythema; 3, moderate erythema (definite redness) with edema; 4, strong erythema (very intense redness) with edema. In a second study, three subjects had three patches containing 1% SLS and three water patches applied to the mid-back for 24 h. Patches were removed, scored and tape stripped as above. In a third study, two individuals were tape stripped on normal skin on the upper back at three adjacent sites as above. RNA harvested in these last two studies was used in the DNA microarray experiments described below.

Materials and reagents Adhesive tape was purchased from Adhesives Research (Glen Rock, Pennsylvania) in bulk rolls. These rolls were custom fabricated into small circular discs, 17 mm in diameter, by Diagnostic Laminations Engineering (Oceanside, California). Total spleen RNA was purchased from Ambion. "RNeasy" RNA extraction kit and Sensiscript Reverse Transcriptase kit were purchased from Qiagen (Valencia, California). PCR primers and probes (TaqMan Pre-Developed Assay Reagents) and TaqMan Universal Master Mix, which included all buffers and enzymes necessary for the amplification and fluorescent detection of specific cDNAs, were purchased from Applied Biosystems (Foster City, California). Total mRNA was amplified using the MessageAmp aRNA kit purchased from Ambion Inc. (Austin, Texas). Human Genome U133A DNA chips were purchased from Affymetrix Inc. (Santa Clara, California).

Isolation of RNA The RNA within skin cells adherent to the four tapes used to harvest a site was pooled by simultaneously extracting the tapes in a volume of buffer RLT (supplied with RNeasy kit). Extraction was performed using the manufacturer's directions and included a Proteinase K digestion, sonication of tapes and "on-column" DNase I digestion. RNA was eluted in 100 μm of sterile, RNase-free water. Extraction of biopsies was performed with the same kit according to the manufacturer's instructions.

Quantitative RT-PCR Ten μL of RNA was reverse transcribed (RT) into cDNA with the Sensiscript Reverse Transcriptase kit and random hexamers in a final volume of 20 μL according to the manufacturer's directions. The reaction was diluted 5-fold with sterile, nuclease-free water (Ambion) for use in the subsequent amplification/detection reaction. For each specific mRNA detection, three replicate RT^+ reactions and one RT^- (no RT; negative control) reaction were performed. Two amplification/detection reactions were done on each RT^+ reaction to yield a total of six independent determinations of the threshold value (C_t; discussed below). All RT^- reactions were amplified using two replicates and were negative (data not shown).

Quantitation of RNA mass recovered with adhesive tape and biopsy The amount of RNA recovered by tape is too small (in most samples) to detect by UV. We have also found that contaminants in the adhesive co-purify with the RNA and interfere with UV and fluorometric detection. We therefore estimated the RNA mass recovered from tapes by using quantitative RT-PCR with reference to a standard curve ($C_{t,actin}$ *versus* log[RNA]; Applied Biosystems, 2001) created from commercially purchased human spleen total RNA. Spleen RNA was treated with DNase I and purified with the Qiagen RNeasy kit following the manufacturer's instructions. Purified standard RNA was quantified spectroscopically using O.D. 260. The standard curve was constructed using four concentrations of RNA from 0.01 to 1 μg per mL. Each RNA standard was reverse transcribed in triplicate and each RT reaction amplified once to yield three replicates per standard concentration. Amplification and detection of unknowns was accomplished as described below using β-actin mRNA as the quantified marker. Experimental samples were reverse transcribed in triplicate and each RT reaction amplified in duplicate to yield a total of six replicates. The average $C_{t,actin}$ of these six replicates was used to calculate the concentration of RNA in the unknown with reference to the standard curve. The accuracy of this method relies on the relative amount of β-actin mRNA to total RNA in the epidermis being similar to that in human spleen. If the relative amount of β-actin mRNA to total RNA is different between the two tissues then our mass data will be similarly affected. Therefore, we describe all tape-harvested RNA mass calculations as estimates to reflect this uncertainty. RNA recovered from biopsies was quantified fluorometrically with the RiboGreen RNA Quantitation Reagent (Molecular Probes, Eugene, Oregon).

Amplification and detection of specific mRNA Specific mRNAs were converted to cDNA as described above. Specific cDNAs were semi-quantified using gene-specific primer/probes (5′-nuclease assay) and fluorescence detection. Amplification and detection assays were performed using TaqMan Pre-Developed Assay Reagents (PDAR; Applied Biosystems) on an Applied Biosystems 7000 Sequence Detection System. IL-1β mRNA was semi-quantitated relative to β-actin in a multiplex assay where β-actin and IL-1β mRNA were detected simultaneously in the same tube. Similarly, IL-8 was semi-quantitated by multiplex PCR with β-actin. The data from these experiments are summarized in Table I and individual subject data are reported in Online Tables S1–S5 and S7. All data were confirmed by singleplex PCR assays where IL-8, IL-1β and β-actin were separately assayed in individual tubes (data not shown). GAPDH was semi-quantitated relative to β-actin using a singleplex format. Thermal cycling conditions were: prior to cycling, 2 min at 50°C, then 10 min at 95°C; then 40 cycles at 95°C for 15 s and 60°C for 60 s. Threshold detection was set at 0.2 for all assays.

Semi-quantitation of mRNA using the $\Delta\Delta C_t$ method and calculation of fold-increase relative to a control sample In this work, we use the comparative or $\Delta\Delta C_t$ method of calculating relative gene expression levels between two samples. In the $\Delta\Delta C_t$ method the levels of IL-1β, IL-8, and GAPDH mRNAs are assayed quantitatively and normalized to β-actin mRNA to create a ratio of the RNA of interest (i.e., IL-1β, IL-8, GAPDH) to β-actin mRNA for each RNA sample. This ratio is then further normalized to a control sample ("calibration"; Applied Biosystems, 2001). This relative level of the RNA of interest to β-actin in a sample is given by the equation $k2^{-(\Delta Ct)}$ where "k" is an unknown constant related to the PCR conditions and the fluorescent probe; because k is unknown, the absolute ratio in a sample is also unknown. ΔC_t is equal to the difference between the experimentally derived C_t values for the mRNA of interest and β-actin mRNA (for instance, $\Delta C_{t,GAPDH} = C_{t,GAPDH} - C_{t,actin}$ for GAPDH normalized to actin). Although an absolute mRNA ratio cannot be determined for a sample with only C_t data, the relative ratio between two samples can be determined. This is illustrated below using, as an example, GAPDH as the

mRNA of interest, β-actin mRNA as the normalizing marker and calibration of the SLS sample to the normal skin sample. The ratio of GAPDH to actin mRNA in an SLS-treated sample is given by

$$\left(\frac{mRNA_{GAPDH}}{mRNA_{\beta-actin}}\right)_{SLS} = k2^{-\Delta C_{t,SLS}} = k2^{-(C_{t,GAPDH}-C_{t,actin})_{SLS}} \quad (1)$$

Likewise the ratio of GAPDH mRNA to actin mRNA in the normal (control) skin sample is given by

$$\left(\frac{mRNA_{GAPDH}}{mRNA_{\beta-actin}}\right)_{normal} = k2^{-\Delta C_{t,normal}} = k2^{-(C_{t,GAPDH}-C_{t,actin})_{normal}} \quad (2)$$

By division of the first equation by the second, simplification, and realization that under identical PCR conditions the constants k are equal, it can be shown that

$$\left(\frac{mRNA_{GAPDH}}{mRNA_{actin}}\right)_{SLS} \Big/ \left(\frac{mRNA_{GAPDH}}{mRNA_{actin}}\right)_{normal} = 2^{-\Delta\Delta C_{t,SLS}} \quad (3)$$

where $\Delta\Delta C_{t,SLS} = \Delta C_{t,SLS} - \Delta C_{t,normal}$ and ΔC_t's are defined above. The C_t values are the experimentally determined number of PCR cycles required to achieve a threshold fluorescence (statistically significant increase in fluorescence over background) for the RNA of interest and β-actin mRNA (Gibson *et al*, 1996; Held *et al*, 1996). Key to the comparative method and accuracy of Equation (3) is the amplification efficiency of the mRNA being quantified. The efficiency of PCR must be very similar if not identical for the amplicons being assayed. In order to reassure ourselves that this is true in this study we initially tested all the primer/probe sets at four different spleen RNA concentrations. In a plot of C_t *versus* log[RNA mass] the slope of the line is a function of the efficiency of amplification (Applied Biosystems, 2001). In these experiments, the slopes were essentially identical and Equation (3) accurately portrays the relative ratios of our mRNAs (data not shown).

RT-PCR data analysis Experimental data are reported as the number of cycles (C_t) required to achieve a threshold fluorescence. Each reported C_t is the mean of six replicate measurements. Calibrated fold-change calculations are made using Equation (3). A calibrated mRNA ratio is considered to be significantly different (>95% confidence interval) than the control if the two standard deviation range of fold-change does not include the value of 1, which is the defined value of the calibrator ($\Delta\Delta C_t$ for the calibrator is equal to 0, with a consequent fold-change relative to itself equal to 2^0). The significance of ΔC_t values (Online Tables S3, S4, and S7) was determined by applying a two-sided, paired *t* test. In some normal skin samples, IL-8 or IL-1β mRNA is undetectable. In these cases, we report an estimated lower limit of fold-change based on a lower limit of detection ($C_t = 37$). It is our experience that the practical limit of detection for real-time PCR is 37 cycles. When the threshold number of PCR cycles (C_t) extends beyond 37, C_t values become highly variable or fluorescence does not achieve a threshold value. With such data, we assign a value of $C_t = 37$ and consider the mRNA undetectable. To illustrate a calculation of the lower limit of fold-change at >95% confidence we use data from subject 7. Subject 7's tape sample of an SLS-treated site has a $(\Delta Ct, IL\text{-}8)_{SLS} = -0.68 \pm 0.08$. The control $\Delta C_t = (C_{t,IL\text{-}8} - C_{t,actin})_{normal} = (C_{t,IL\text{-}8} - 29.8)$; because the IL-8 mRNA was undetectable we assign it a value of 37, hence $\Delta C_{IL\text{-}8}$ in the control sample is 37–29.8 = 7.2 (± 0.13), the value reported in Online Table S4. We can now calculate the calibrated fold-change of IL-8 in the SLS sample as $2^{-(\Delta\Delta Ct)} = 2^{-(-0.68-7.2)} = 236$. The lower limit of the >95% confidence interval, however, is given as $2^{-(\Delta\Delta Ct + 2SD)} = 2^{-(-7.88+2\times0.18)} = 189$, the value reported in online Table S4 (0.18 is the error in $\Delta\Delta C_t = ((0.08)^2 + (0.13)^2)^{1/2}$).

T7 linear RNA amplification mRNA was amplified and biotin labeled using a MessageAmp aRNA kit purchased from Ambion Inc, according to the manufacturer's instructions. Typical yields of aRNA obtained from two rounds of amplification ranged from 30 to 80 μg.

Hybridization of biotinylated mRNA targets to Affymetrix GeneChips, staining, data acquisition and data analysis Hybridization and staining were performed according to the manufacturer's instructions; a detailed protocol is available at the manufacturer's website.

DNA microarray data analysis The DNA microarray data reported here were globally normalized and background subtracted with the Affymetrix MAS 5.0 software package and modeled with the dChip program of Li and Wong (2001). Statistical analyses were performed with the Cyber-T statistical program available on line at www.igb.uci.edu. The posterior probabilities of differential expression (PPDE) values (ranging from 0 to 1) for each gene were based on estimates of experiment-wide false positive and negative levels calculated by Cyber-T according to the methods of Allison and Gadbury (2002) as described by Hung *et al* (2002) and Baldi and Hatfield (2002).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID22729/JID22729sm.htm

Table S1. Fold-change of GAPDH/β-actin mRNA ratio in tape and biopsy samples of SLS-treated and water-treated skin calibrated to normal skin

Table S2. Summary of fold-changes in IL-1β/β-actin mRNA ratio in SLS-treated and water-treated skin relative to normal skin

Table S3. ΔC_t values for IL-1β mRNA in normal, water-treated and SLS-treated skin samples recovered by tape and biopsy

Table S4. ΔC_t values for IL-8 mRNA in normal, water-occluded and SLS-occluded skin

Table S5. Summary of fold-changes in IL-8/β-actin mRNA ratio in SLS-treated and water-treated skin relative to normal skin

Table S6. Functional grouping of top 100 differentially expressed genes between untreated and SLS treated conditions with p-values less than 1.4×10^{-10} and PPDE values greater than 0.99

Table S7. $\Delta C_{t,\ GAPDH}$ values in SLS-treated, water-treated and untreated skin

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